

## **REMARKS**

Entry of the above amendments and reconsideration of this application is respectfully requested. Claims 44, 96, 98, 133-135 and 137-146 have been withdrawn from consideration. Claim 37 has been amended to more clearly describe the serum. Support for such an amendment is found generally throughout the application including claim 37 prior to this amendment. Thus, no new matter has been added by these amendments. Upon entry of these amendments, claims 34-38, 41-43, 95, 97, 110-114, 123-132 and 136 remain pending and under consideration. In view of the amendments and the following remarks, it is believed that all rejections are overcome and that this application is in condition for allowance.

### **Interview Summary**

The undersigned attorney would like to thank Examiner Bristol for the courtesy of the telephonic interview on August 12, 2010. The biological deposit and obviousness rejections were generally discussed.

### **Claim Objections**

Claims 41 and 112 were objected to but no reasoning was given. Clarification is respectfully requested.

### **Double Patenting**

Claims 34-38, 42, 43, 95, 110, 111, 113, 114, 123-132 and 136 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 98-109 of U.S. Patent Application No. 09/852,547. Applicant notes that this is a provisional double patenting rejection for which the M.P.E.P. at § 1504.06 Double Patenting provides as follows:

If a provisional double patenting rejection (of any type) is the only rejection remaining in two conflicting applications, the examiner should withdraw that rejection in one of the applications (e.g., the application with the earlier filing date) and permit the application to issue as a patent. The examiner should maintain the provisional double patenting rejection in the other application which rejection will be converted into a double patenting rejection when the first application issues as a patent. If more than two applications conflict with each other and one is allowed, the remaining applications should be cross rejected against the others as well as the allowed application. For this

type of rejection to be appropriate, there must be either at least one inventor in common, or a common assignee. If the claims in copending design applications or a design patent and design applications have a common assignee but different inventive entities, rejections under 35 U.S.C. 102(e), (f) and (g)/103(a) must be considered in addition to the double patenting rejection. See MPEP Section 804, Section 2136, Section 2137 and Section 2138.

Accordingly, should the Examiner find the present claims allowable in view of the above amendments and arguments herein, Applicants respectfully request withdrawal of this provisional rejection in the instant case. Otherwise, Applicants will address this rejection upon indication of allowable subject matter in either application.

#### **Claim Rejections Under 35 U.S.C. § 112, first paragraph**

Claims 123, 125, 126, and 128 stand rejected under 35 U.S.C. § 112, first paragraph, because the Examiner contends that it is unclear if a cell line for MTW9/PL2, MCF-7K, or H-301 is publicly available.

While not conceding to the validity of these rejections, Applicants have elected to cancel the cell lines MTW9/PL2, H-301 and MCF-7K from the rejected claims in an effort to expedite prosecution. Thus, this rejection is rendered moot.

#### **Claim Rejections Under 35 U.S.C. § 112, second paragraph**

Claim 37 stands rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for the recitation of "wherein the nutrient medium further includes non-heat inactivated serum." Claim 37 has been amended to specify that the serum has not been subjected to heat inactivation. Applicants submit that such an amendment renders the claim definite. Withdrawal of this rejection is therefore respectfully requested.

#### **Claim Rejections Under 35 U.S.C. § 103(a)**

Claims 34, 37, 38, 42, 43, 95, 97, 123-132 and 136 stand rejected under 35 U.S.C. §103(a) as being unpatentable over Furuya et al (Cancer Research, December 1989, Vol. 49, pp 6670-6674) in view of Hoffman ('The Biochemistry of Clinical Medicine', 1970, pages 48-55). The Examiner contends that Furuya et al. teach that a bovine serum albumin fraction containing globulin remnants inhibited cell growth but that globulin-free bovine serum albumin did not

inhibit cell growth. The Examiner then reasons that one skilled in the art would reasonably conclude that serum globulins were the cause of the growth inhibition. The Examiner also contends that Furuya et al. teach that estradiol can overcome this inhibition. The Examiner acknowledges that Furuya et al. fail to teach growth stimulation by estrogen in the presence of IgA or IgM but suggests that Hoffman et al. provides evidence that these components are present within the globulin fraction of serum. The Examiner therefore reasons that one skilled in the art would be motivated to test the inhibitory contributions of serum globulin proteins to identify the factor(s) responsible for growth inhibition. Applicants respectfully disagree.

Furuya et al. teach the use of a globulin free serum albumin to look at the direct effects of estradiol and Tamoxifen on MCF-7 cells. Furuya et al. teach against the use of a serum that includes globulin remnants. Thus, Furuya et al. fail to teach the use of a medium including immunoglobulins of any kind. Each independent claim requires a nutrient medium including an immunoglobulin. "All words in a claim must be considered in judging the patentability of that claim against the prior art." MPEP § 2143.03 (citing *In re Wilson*, 424 F.2d 1382, 1385, 165 USPQ 494, 496 (CCPA 1970)). Using the same reasoning, Furuya et al. teach away from the use of a nutrient medium including an immunoglobulin. An invention is not obvious if the cited references teach away from that invention. See MPEP § 2145(X)(D)(2). As well, using a nutrient medium including a cell growth inhibitor of any kind would render the methods taught by Furuya et al. unsatisfactory/inoperable for its intended purpose. The proposed modification cannot render the prior art unsatisfactory (or inoperable) for its intended purpose. See MPEP § 2143.01(V). The purpose of the method taught by Furuya et al. is to look at the effects of estradiol and Tamoxifen on MCF-7 cells in a medium lacking components that can inhibit the growth of the MCF-7 cells. Conversely, the present claims specifically require the presence of cell growth inhibitors in the nutrient medium containing cancer cells.

Turning now to Hoffman et al., this reference merely shows the distribution of IgG, IgA, IgM and IgD in normal serum. Even assuming, *arguendo*, that the normal serum depicted in Fig. 4 of Hoffman et al. is the same as the bovine serum albumin V fraction used by Furuya et al., there is no teaching or suggestion that these immunoglobulins, and specifically IgA and IgM as presently claimed, are responsible for the growth inhibition suggested by Furuya et al. Indeed,

BSA-V is generally known in the art as being a very crude fraction containing hundreds of serum proteins and many other impurities. One skilled in the art could not ascertain that any growth inhibitory function was the result of IgA and IgM prior to the Applicants' discovery. No specificity can be obtained by using crude fractions. For any growth inhibitor identification to be accomplished, these inhibitors must be purified and further tested to show they are responsible for cell growth inhibition. These immunoglobulins, through many purification steps, were eventually shown to be responsible for growth inhibition of cancer cells. See the present application at Example 17. Such an association was not possible prior to these experimental results.

In addition to the above, Applicants note that Furuya et al. was published in 1989 and Hoffman et al. was published as early as 1954 (more recently in 1970). If the identification of IgA and IgM was an obvious undertaking as suggested by the Examiner, Applicants submit that such a discovery would have taken a much shorter period of time. As discussed in Example 17, prior documents have suggested a serum-borne growth inhibiting factor(s) but had yet to identify its existence. More specifically, Soto et al. (*J. Steroid Biochem. Molec. Biol.* Vol. 43, No. 7, pp. 703-712 (1992)) attempted such an undertaking and published their unsuccessful results in 1992. Thus, almost three full years after the Furuya et al. publication, those skilled in the art were unable to purify these serum-borne growth inhibiting factor(s). In fact, Soto et al. presents evidence which would lead one skilled in the art away from IgA and IgM as the growth inhibiting factors. Soto et al. state that “[e]strocolyone-I activity seems to be due to a protein of an apparent native  $M_w$  of 70-80 kDa...” See Soto et al. at page 703, Summary, last full sentence. It is well known in the art that IgA (dimer) has a molecular weight of 320kDa and IgM has a molecular weight of 900kDa, which are both well outside the range stated by Soto et al. Moreover, Soto et al. state that “[f]urther purification of estrocolyone-I is being pursued, a step necessary for a mechanistic understanding of its action and that of estrogens on the control of cell proliferation.” See Soto et al. at page 711 last sentence. Given the above, the Soto et al. reference is being filed concurrently herewith as evidence showing the failure of others in achieving the presently claimed methods.

In view of the above, Applicants respectfully submit that a proper case of obviousness has not been established. Withdrawal of this rejection is respectfully requested.

### **Conclusion**

In view of the foregoing, it is submitted that all rejections have been overcome. Withdrawal of the rejections and allowance of the claims are solicited.

### **Request for Interview**

In the event that the Examiner finds any reason that the application cannot be allowed in its present form, the Applicant wishes to conduct an interview with the Examiner prior to any further action in order to provide an opportunity for coming to agreement upon allowable claims. To arrange the interview, the Examiner is requested to call the undersigned attorney at the telephone number given.

Respectfully submitted,

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By



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## A PLASMA-BORNE SPECIFIC INHIBITOR OF THE PROLIFERATION OF HUMAN ESTROGEN-SENSITIVE BREAST TUMOR CELLS (ESTROCOLYONE-I)

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**Summary**—Charcoal-dextran stripped serum/plasma supplemented media specifically inhibit the proliferation of estrogen-sensitive cells in culture conditions; estrogens cancel this effect. Here, we further characterize this phenomenon using human estrogen-sensitive breast cancer MCF<sub>7</sub> cells and human serum/plasma. The serum/plasma-borne inhibitory activity (estroclyone-I) is a non-dialyzable, heat-stable (60°C × 2 h), protease-sensitive macromolecule and it is not extractable by organic solvents. Estroclyone-I activity is retained after dialysis against 6 M urea or 10–100 mM dithiothreitol; however, simultaneous treatment with 6 M urea and 10–100 mM dithiothreitol completely abolishes its inhibitory activity. The inhibitory effect of serum is not due to serum albumin, nor to estrogen trapping by albumin or by sex hormone-binding globulin. Substantial purification was achieved by a combination of chromatographic techniques (dye-affinity, ion exchange, hydrophobic interaction chromatography). Estroclyone-I activity seems to be due to a protein of an apparent native M<sub>w</sub> of 70–80 kDa and an isoelectric point of 4.5–4.8.

### INTRODUCTION

The regulation of cell proliferation by estrogens is incompletely understood. This subject is currently being explored under three different working hypotheses. The direct-positive hypothesis that proposes that estrogens *per se* trigger the multiplication of their target cells [1–4]. Alternatively, the indirect-positive hypothesis that proposes that estrogens induce the synthesis and/or release of growth factors [5] which, in turn, cause proliferation of their target organs. The autocrine mechanism [6], a variation of this latter hypothesis, proposes that estrogens would induce the synthesis and secretion of a variety of growth factors by their own target cells. In turn, these autocrine growth factors would elicit the proliferation of the same cells which secreted them [7, 8]. In contrast to predictions of the positive hypotheses, several reports showed that cells that are estrogen-dependent when inoculated into animals proliferate maximally in serumless medium regardless of the presence of estrogen [9–13]. Because of these data, we have been engaged in exploring the indirect-negative hypothesis: it proposes that estrogens neutralize the action

of blood-borne specific proliferation inhibitor(s) [10, 11, 14–16].

MCF<sub>7</sub> cells were chosen as a model because (a) they grow as estrogen-dependent tumors in athymic mice [17–19], (b) charcoal-dextran stripped human serum inhibits their proliferation in a dose-dependent pattern [10, 11], and (c) estrogens cancel the inhibitory effect of serum at physiological concentrations. Because these cells also proliferate maximally in serumless medium regardless of the presence of estrogens, we concluded that serum contains a specific inhibitor of the proliferation of estrogen-sensitive cells, estroclyone-I; estrogens would merely neutralize this inhibitory effect [10, 11, 16]. Here, we describe the characterization and partial purification of estroclyone-I.

### METHODS

#### Cell culture procedures

A clonal population of the human breast cancer MCF<sub>7</sub> cell line was used in these experiments [10]. To assess the inhibitory effect of serum/plasma or partially purified serum/plasma fractions,  $5 \times 10^4$  cells were seeded in Costar 3512 Multiplates in 5% fetal bovine

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serum (FBS); they were allowed to attach for 24 h. Then, 1 ml of Dulbecco's modification of Eagle medium (DME) plus 2  $\mu$ g transferrin (T)/ml and 100 ng insulin (I)/ml (IT-DME) alone or IT-DME plus (i) charcoal-dextran stripped (CD) serum or (ii) partially purified preparations were placed in each well. Each preparation was tested by triplicate in the presence and absence of 30 pM 17 $\beta$ -estradiol ( $E_2$ ). Phenol-red free media were used in all experiments [20]. At selected time intervals cells were lysed [10% Zapoglobin (Coulter Electronics, Hialeah, FL)] and nuclei counted on a Coulter Counter Model ZF<sub>1</sub>. Previous to testing the inhibitory potency of partially purified estrocolonyone-I, fractions were concentrated by ultrafiltration (YM30 Amicon membranes, nominal cutoff = 30 kDa) and dialyzed through cellulose membranes (nominal cutoff = 25 kDa) against a buffer suitable for tissue culture (TC buffer: 100 mM NaCl, 25 mM HEPES, pH 7.4). Estrogens were removed from serum and from partially purified plasma fractions by charcoal-dextran stripping [10, 11].

#### *Purification procedures*

Plasma coagulation factors were removed by precipitation with 80 mM BaCl<sub>2</sub> [21]. Matrex-green chromatography (Amicon Corp., Lexington, MA) of BaCl<sub>2</sub>-treated plasma was used to remove human serum albumin (HSA). The column was equilibrated with 25 mM bistris-propane (BTP), pH 7.0, and 0.6 ml plasma were loaded/ml packed gel. The breakthrough was used for estrocolonyone-I purification and the bound fraction was eluted with 2 M NaCl, 25 mM BTP, pH 7.0. This fraction was used in protease digestion experiments. For anion exchange chromatography, start buffers were 50 mM piperazine pH 6.0 and BTP, pH 7.0 and 9.5. Limit buffers contained 1 M NaCl. 50 to 200 mg Matrex-green breakthrough protein were injected into a FPLC MonoQ HR10/10 column equilibrated with start buffer; separation was carried out by linear gradient elution. For hydrophobic interaction chromatography, samples were equilibrated in start buffer, injected into a phenyl-Superose HR5/5 column and resolved by linear gradient [50 mM sodium phosphate buffer containing 0.4 to 2 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> as start buffer and 20% acetonitrile-50 mM Na phosphate pH 7.0 as limit buffer]. To determine the pI of estrocolonyone-I, chromatofocusing was performed in a MonoP HR25/5 column; pH gradients from 7.0 to 4.0 and from 5.5 to 4.0 were

generated with 10% Polybuffer 74. Separation by gel filtration in FPLC Superose 12 HR 16/50 (bed volume: 98 ml) and in Superdex 200 XK 26/60 (bed volume: 320 ml) columns were used for assessing the native molecular weight of estrocolonyone-I. Molecular weight standards were used to monitor the elution pattern. FPLC instruments and reagents were from Pharmacia-LKB (Piscataway, NJ). All buffers contained 1 mM benzamidine, a protease inhibitor.

#### *Protease hydrolysis*

Charcoal-dextran stripped rooster serum was incubated at 37°C for 2 h with and without 0.25 mg trypsin/ml (8000 BAEE U/mg). 5000 U soybean trypsin inhibitor/ml were added to stop the proteolytic effect; after dilution to 10% with IT-DME, sera were tested for estrocolonyone-I activity. Proteases were removed from human plasma by Matrex-green chromatography; aliquots were dialyzed against 50 mM CaCl<sub>2</sub>-50 mM Tris-HCl, pH 8.0 for alpha-chymotrypsin and against 1 mM EDTA, 1 mM dithiothreitol (DTT), 50 mM piperazine, pH 6.3 for papain digestion. Aliquots were incubated at 37°C for 16 h with 1 ml plain agarose or 100 U of protease coupled to 1 ml agarose (Sigma Chemical Co, St Louis, MO). The digested preparations were dialyzed against TC buffer, diluted with IT-DME and assayed for estrocolonyone-I activity.

#### *Hexane, dichloromethane and acetone extraction of serum*

5 ml CDHuS aliquots were acidified with 0.5 ml 10 N HCl and extracted with 5 ml hexane or dichloromethane; the solvent phase was evaporated and resuspended in 5 ml of 2 mg HSA/ml in IT-DME. 5 ml acetone cooled at -20°C were added dropwise to 5 ml CDHuS cooled at 4°C; after centrifugation (2000 g  $\times$  15 min) the precipitate was resuspended and dialyzed against TC buffer. The supernatant was evaporated in a Speed Vac concentrator (Savant Instruments, Long Island, NY) and resuspended in 2 mg/ml HSA in IT-DME.

#### *Acid extraction of serum*

To determine whether or not estrocolonyone-I activity was due to chalone-like oligopeptides [22], CDHuS was dialyzed against 50 mM NH<sub>4</sub>HCO<sub>3</sub>, lyophilized, resuspended in water and extracted with 2 M acetic acid. Precipitated proteins were removed by centrifugation at 100,000 g and the supernatant was dialyzed

twice against 10 vol of 0.5 M acetic acid. These supernatants were dialyzed against TC buffer, brought to original volume and assayed for cell proliferation at concentrations of 5 and 10%.

#### Denaturation studies

The effect of urea and DTT on estrocolonyone-I activity was studied as follows: (a) urea crystals (ultrapure grade, Schwarz-Mann Biotech, Cleveland, OH) were added to 10 ml of CDHuS to a final concentration of 6 M. Comparable aliquots were treated with (b) 10 mM DTT, (c) 100 mM DTT, (d) 6 M urea plus 10 mM DTT and (e) 6 M urea plus 100 mM DTT. After 2 h incubation at room temperature, these preparations were dialyzed twice at 4°C for 16 h against 10 vol of 25 mM BTP containing: (a) 6 M urea, (b) 10 mM DTT, (c) 100 mM DTT, (d) 6 M urea plus 10 mM DTT and (e) 6 M urea plus 100 mM DTT, respectively. Precipitated material was removed by centrifugation and each preparation was dialyzed against TC buffer.

#### Cell cycle analysis

Cells were harvested in 0.2% triton X-100 in Hanks' balanced salt solution containing 2 µg 4'-6-diamidino-2-phenylindol dihydrochloride/ml [23].  $10^4$  cells were analyzed by flow cytometry with a 350/356 krypton laser [24].

#### HSA

Depending on the concentration range expected, two methods were used. Levels between 5 and 60 mg/ml were measured by the standard bromocresol green method [25]. Levels below 5 mg/ml were measured by a double antibody ELISA based on the assay described for fibronectin by Rennard *et al.* [26]. Briefly, plate coating was performed using 100 ng HSA/0.2 ml for 24 h at 4°C; simultaneously, the first antibody (rabbit anti-HSA 1:4000) was incubated with antigen in liquid phase. After washing the plates and saturating plastic binding sites with 0.5% gelatine, the HSA-first antibody mixture was placed into the coated wells, incubated for 2 h at 22°C; after washing, 0.2 ml of alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (Sigma) (1:500) were added for 2 h, washed and incubated for 30 min with 0.2 ml of 1 mg/ml *p*-nitrophenyl phosphate; the reaction was stopped with 0.05 ml 3N NaOH and read at 405 nm. This indirect ELISA was very sensitive (10 ng/ml) and reproducible. Anti-HSA serum was obtained as described previously [27].

#### Sex hormone binding globulin (SHBG)

Purification was done by ligand affinity chromatography [28]; the column breakthrough was used as SHBG-free plasma.

Unless specified otherwise, all chemicals were purchased from Sigma; TGF- $\beta$  was from Collaborative Research (Waltham, MA). Porcine insulin was a gift from the Eli Lilly Co. (Indianapolis, IN).

#### RESULTS

##### Effects of serumless medium, CDHuS and $E_2$ on proliferation

Cell proliferation curves revealed the following pattern: proliferation rates were maximal for the first 48 h regardless of the presence of  $E_2$  or CDHuS and later, they decreased significantly only in CDHuS supplemented medium [Fig. 1(A)]. At 96 h, 95% of cells in CDHuS had a  $G_0/G_1$  DNA content [Fig. 1(B), bottom panel] while in IT-DME with or without  $E_2$  and CDHuS plus  $E_2$  cells proliferated maximally; the DNA content distribution was 57% in  $G_0/G_1$ , 36% in S, and 7% in  $G_2$  [Fig. 1(B), top panel].

##### Estrocolonyone-I bioassay

The best estimate of proliferative activity is doubling time ( $t_D$ ) measured during the exponential phase [29]. For practical reasons, a single time point assay was developed based on the evidence presented in Fig. 1. This assay measures the cell number achieved by an inoculum of  $5 \times 10^4$  cells after 96 h of exposure to plasma fractions added to IT-DME both in the presence and absence of 30 pM  $E_2$ . A control for maximal proliferation yield is given by a similar cell inoculum grown in IT-DME. A standard curve of estrocolonyone activity is simultaneously run using serial dilutions of CDHuS. One unit of estrocolonyone-I activity is defined as the amount necessary to inhibit a population doubling of an inoculum of  $5 \times 10^4$  cells/well in 1 ml of IT-DME after 96 h of exposure. Estrocolonyone-I was measured as described above because cell proliferation is an exponential function; therefore, conditions had to be set to allow maximal proliferation rates in the absence of inhibitor as depicted in Fig. 1(A) (cells cultured in IT-DME proliferated maximally as did cells in CDHuS plus  $E_2$ ). One estrocolonyone-I unit is contained in 3 mg serum protein (5% serum). Estrocolonyone-I content is measured by serial dilution; the assay measures the lowest dilution to show an activity equal to 1 U.



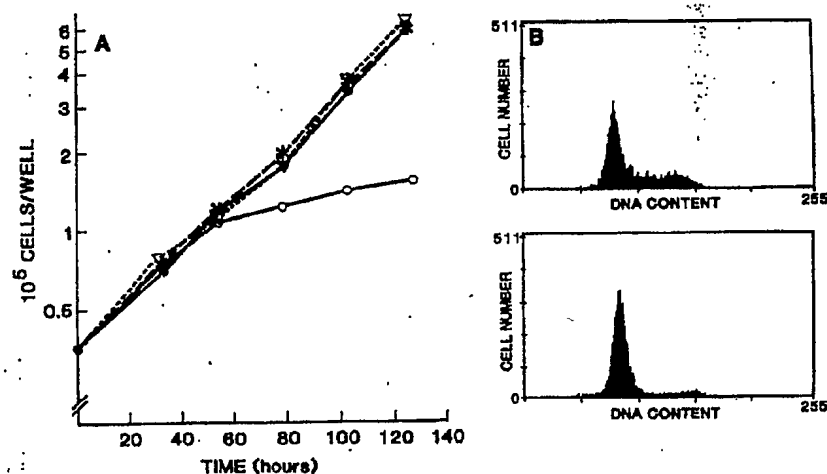


Fig. 1. (A) Cell proliferation curves of cloned MCF7 cells grown in: IT-DME with (\*—\*) and without 30 pM  $E_2$  ( $\nabla$ — $\nabla$ ); 10% CDHuS, both in the presence (●—●) and absence (○—○) of 30 pM  $E_2$ . Values indicate the mean of 4 experimental measurements; because SD was less than or equal in magnitude to the size of the plotted data point bars were not plotted. (B) Cell cycle profile by flow cytometry. Upper panel, cells in 10% CDHuS plus 30 pM  $E_2$ . Lower panel, cells in 10% CDHuS show a  $G_0/G_1$  profile.

#### On the nature of estrocolonyone-I activity

To assess whether or not estrocolonyone-I is a small molecule, serum was extensively dialyzed against 100 mM NaCl, 25 mM HEPES, pH 7.4; membranes of nominal  $M_w$  cut-off ranging from 3.5 to 25 kDa retained estrocolonyone-I activity (not shown). These results indicate that either estrocolonyone-I is not a small molecule, or that it is tightly bound to a macromolecule. In addition, organic solvents such as acetone (Table 1), hexane and dichloromethane failed to

extract estrocolonyone-I activity from serum; this observation suggests that estrocolonyone-I is not a steroid-like molecule.

Acid extraction of CDHuS was performed to assess whether or not estrocolonyone-I activity was due to small chalone-like peptides; these peptides bind to macromolecules and are released from them by acid treatment [22]. Estrocolonyone-I, unlike chalones, was retained in the macromolecular compartment (Table 1).

Dialysis against 6 M urea, 25 mM HEPES, pH 7.4 also retained estrocolonyone-I activity (not

Table 1. Characterization of estrocolonyone-I: effect of serum components on the proliferation of MCF7 cells

Source	Cell number <sup>a</sup>		$E_2 + / E_2 -$ ratio	Control <sup>b</sup>
	Without $E_2$	0.3 nM $E_2$		
5% CDHuS <sup>1</sup>	130 ± 7	389 ± 31	3.0	375 ± 2
25 kDa membrane cutoff <sup>1</sup>	216 ± 4	482 ± 2	2.2	381 ± 18
6 M urea <sup>2</sup>	83 ± 2	217 ± 27	2.6	375 ± 2
6 M urea, 10 mM DTT <sup>2</sup>	370 ± 12	381 ± 18	1.0	375 ± 2
100 mM DTT <sup>2</sup>	215 ± 1	521 ± 5	2.4	520 ± 9
2 M acetic acid extract <sup>3</sup>	67 ± 2	160 ± 2	2.4	424 ± 13
Acetone extract <sup>3</sup>	333 ± 12	351 ± 8	1.1	310 ± 3
Acetone pellet <sup>4</sup>	125 ± 9	320 ± 8	2.6	310 ± 3
30 nM SHBG <sup>5</sup>	133 ± 8	165 ± 10	1.2	318 ± 3
5% SHBG-free plasma <sup>6</sup>	242 ± 8	472 ± 5	2.0	524 ± 5
2 mg HSA/ml <sup>7</sup>	361 ± 12	389 ± 3	1.1	341 ± 9

<sup>a</sup>Cell number is expressed as cells/well  $\times 10^3 \pm$  SD. <sup>b</sup>Control is the cell yield of a similar inoculum grown simultaneously in IT-DME. <sup>1</sup>5% CDHuS. <sup>2</sup>CDHuS aliquots were treated as indicated; retained proteins were dialyzed against TC buffer and assayed at 3 mg/ml IT-DME. <sup>3</sup>extracts were dried down and resuspended to the original serum volume with IT-DME containing 2 mg/ml HSA (Sigma, Cat No. A8763). <sup>4</sup>pellet was resuspended to the original serum volume with TC buffer, dialyzed against TC buffer and diluted to 5% with IT-DME. <sup>5</sup>SHBG was purified, molarity was calculated from its androgen binding capacity; after dialysis against DB, SHBG was diluted with IT-DME. <sup>6</sup>The affinity column breakthrough was dialyzed against DB and diluted with IT-DME. <sup>7</sup>Sigma, Cat No. A8763.

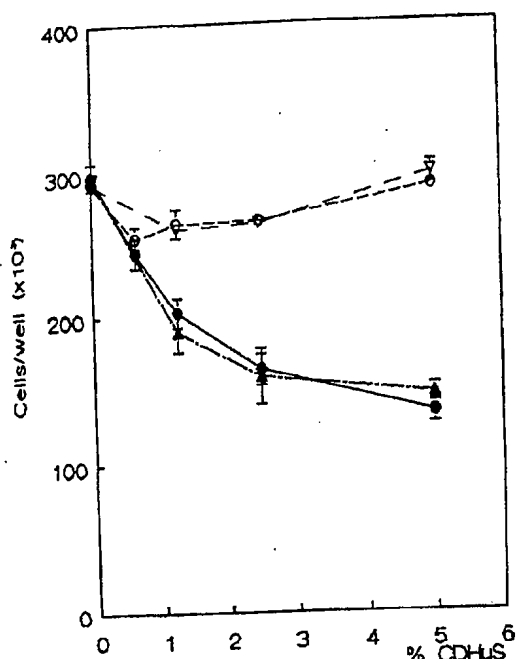


Fig. 2. MCF<sub>7</sub> cell yields were measured after 96 h of exposure to variable concentrations of: (i) CDHuS (normal donor) alone (●—●), or (ii) plus 30 pM E<sub>2</sub> (○—○), (iii) CDHuS (analbuminemic donor) alone (▲—▲), or (iv) plus 30 pM E<sub>2</sub> (▽—▽).

shown). Activity was not affected by dialysis against 10–100 mM DTT, 100 mM NaCl, 25 mM HEPES, pH 7.4; however, dialysis against 6 M urea, 10–100 mM DTT, 25 mM HEPES, pH 7.4 resulted in complete loss of estrocolonyone-I activity (Table 1). These results are consistent with the notion that estrocolonyone-I is a macromolecule; the inactivation effect of DTT only in the presence of 6 M urea suggests that a disulfide bond necessary for estrocolonyone-I activity is either not accessible or it regenerates readily in non-denaturing conditions.

To assess whether or not proliferation inhibition by serum was due to trapping of estro-

gens by the serum proteins SHBG and HSA, these purified proteins were added to IT-DME. Neither inhibited MCF<sub>7</sub> cell proliferation; moreover, SHBG-free plasma obtained by adsorption of SHBG to cortisol-Sepharose retained the estrocolonyone-I activity. TGF- $\beta$  was marginally inhibitory at 0.1 nM but this effect was not cancelled by E<sub>2</sub>. Cell yields were: 118,000  $\pm$  11,000 in IT-DME; 86,000  $\pm$  7000 in IT-DME + TGF- $\beta$  and 72,000  $\pm$  4000 in IT-DME + TGF- $\beta$  + 0.3 mM E<sub>2</sub>.

#### Estrocolonyone-I activity in analbuminemic human serum

To ascertain whether or not estrocolonyone-I activity is due to HSA as proposed by Laursen *et al.* [30], three strategies were implemented: (a) chromatographic separation as reported below, (b) testing the inhibitory activity of commercially obtained HSA (Table 1), and (c) testing the inhibitory activity of serum from an analbuminemic donor [31]. The HSA concentration in analbuminemic serum was <0.1 mg/ml, while in the normal serum used as control the HSA concentration was 40 mg/ml. Figure 2 compares the dose-response curve to normal and analbuminemic CD serum; both curves are superimposable indicating that estrocolonyone-I activity is present in analbuminemic serum.

#### Protease treatment

Human serum contains macromolecular protease inhibitors such as  $\alpha_1$ -anti-trypsin; this precludes the use of trypsin treatment of whole serum to assess whether or not estrocolonyone-I is a trypsin-sensitive macromolecule. CD-avian serum also inhibits the proliferation of MCF<sub>7</sub> cells but, unlike human serum, it does not contain  $\alpha_1$ -anti-trypsin. Trypsin digestion of rooster serum resulted in loss of inhibitory

Table 2. Protease sensitivity of serum estrocolonyone-I activity

Treatment	Control			Enzyme-treated		
	No E <sub>2</sub>	0.3 nM E <sub>2</sub>	E <sub>2</sub> +/E <sub>2</sub> - ratio	No E <sub>2</sub>	0.3 nM E <sub>2</sub>	E <sub>2</sub> +/E <sub>2</sub> - ratio
Trypsin <sup>a</sup>	180 $\pm$ 8	355 $\pm$ 14	2.0	285 $\pm$ 16	324 $\pm$ 24	1.1
$\alpha$ -Chymo-trypsin <sup>b</sup>	91 $\pm$ 3	287 $\pm$ 76	3.2	174 $\pm$ 32	265 $\pm$ 7	1.5
Trypsin <sup>b</sup>	114 $\pm$ 3	259 $\pm$ 13	2.3	168 $\pm$ 33	219 $\pm$ 8	1.3
Papain <sup>b</sup>	144 $\pm$ 2	395 $\pm$ 10	2.7	161 $\pm$ 3	168 $\pm$ 37	1.0

<sup>a</sup>CD-rooster serum was treated with 0.25 mg trypsin/ml, 2 h at 37°C; 5000 U soybean trypsin inhibitor/ml were added, and serum was assayed at 10%.

<sup>b</sup>1 ml agarose (control) or immobilized enzyme (100 U/ml agarose) were incubated with 5 ml Matrex-green fraction; after incubation for 16 h, at 37°C and dialysis samples were treated in culture at 10% dilution. Results are expressed as the mean  $\pm$  SD of a set of three experiments.

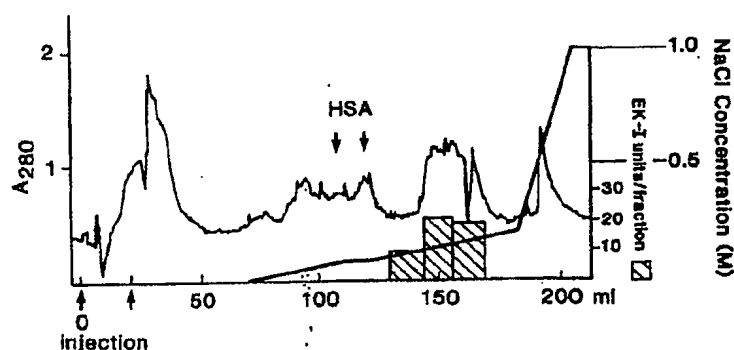


Fig. 3. 50 mg Matrex-green eluate were chromatographed through a MonoQ HR10/10 column. Start buffer: 20% acetonitrile, 2 mM DTT, 1 mM benzamidine, 50 mM piperazine, pH 6. Limit buffer: 1 M NaCl in start buffer.

activity (Table 2). Human plasma was treated with 80 mM  $\text{BaCl}_2$  to remove endogenous proteases and chromatographed through Matrex-green agarose to remove endogenous protease inhibitors. The retained fraction containing 50% of the estrocolonyone-I activity and most HSA was eluted with 2 M NaCl, 25 mM HEPES, pH 7.0, dialyzed, and digested with proteases covalently bound to the agarose gel as described in Methods. Protease-treated human estrocolonyone-I fractions no longer inhibited MCF<sub>7</sub> cell proliferation (Table 2).

#### Separation of estrocolonyone-I and HSA

Estrocolonyone-I and HSA coprecipitated with  $(\text{NH}_4)_2\text{SO}_4$  between 60 and 70% saturation. HSA and estrocolonyone-I were retained by and coeluted from blue-Sepharose. Matrex-green also bound HSA; the breakthrough fraction contained only 15% of the HSA load with an estrocolonyone-I yield of 50%. This fraction was used for further estrocolonyone-I purification. A 1.5- to 3-fold purification was achieved by this procedure.

#### Ion exchange chromatography of the Matrex-green breakthrough

Estrocolonyone-I was adsorbed to cation exchangers at pH 4.0 and to anion exchangers at pH 6.0. Chromatographic titration curves were performed on a MonoQ column; estrocolonyone-I and HSA coeluted at pH 7.0 and 8.0 and were resolved at pH 6.0 and 9.5. Binding at pH 6.0 and adding 20% acetonitrile and 2 mM DTT to the start and limit buffers improved separation (Fig. 3). Estrocolonyone-I activity was eluted between 120 and 170 mM NaCl. In a series of 3 experiments the purification achieved through this step was 30- to 40-fold; the overall purification was 40- to 60-fold. A representative experiment is shown in Table 3.

#### Hydrophobic interaction chromatography (HIC)

Complete estrocolonyone-I adsorption was achieved at 2 M  $(\text{NH}_4)_2\text{SO}_4$ ; estrocolonyone-I was eluted with a linear gradient. This chromatographic step resulted in a 7- to 14-fold purification when performed after Matrex-

Table 3. Estrocolonyone-I purification by anion exchange chromatography

Fraction	Protein content <sup>a</sup>	Specific activity <sup>b</sup>	Total yield <sup>c</sup>	Yield (%) <sup>d</sup>	Purification <sup>e</sup>
$\text{BaCl}_2$ -plasma	155.00	1.33	199.0	100	1.0
Matrex-green breakthrough	50.00	2.00	100.0	50	1.5
MonoQ fraction No. 5	1.38	11.40	15.7	8	7.5
MonoQ fraction No. 6	0.25	80.00	20.0	10	60.0

Purification procedures are described in Methods. Anion exchange chromatography was performed in a MonoQ HR10/10 column; the start buffer was 20% acetonitrile, 2 mM DTT, 1 mM benzamidine, 50 mM piperazine, pH 6, and the limit buffer was 1 M NaCl in start buffer.

<sup>a</sup>Protein content is expressed in mg.

<sup>b</sup>Specific activity is expressed in estrocolonyone-I U/mg protein.

<sup>c</sup>Total estrocolonyone-I yield is calculated as the product of the protein content by the specific activity of the estrocolonyone-I preparation.

<sup>d</sup>Percent yield is calculated as  $100 \times \text{total estrocolonyone-I yield of each purification step} / \text{estrocolonyone-I yield of the starting material (BaCl}_2\text{-plasma)}$ .

<sup>e</sup>Purification is calculated as the ratio between the specific activity achieved after a purification step and the initial specific activity of the starting material.

Table 4. Estrocolonyone-I purification by hydrophobic interaction chromatography

Fraction	Protein content (mg)	Specific activity <sup>b</sup>	Total yield <sup>c</sup>	Yield (%) <sup>d</sup>	Purification <sup>e</sup>
BaCl <sub>2</sub> -plasma	110.00	1.00	110.0	100	1.0
Matrex-green breakthrough	31.96	2.20	70.3	63	2.2
2 M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> supernatant	13.60	5.00	68.0	61	5.0
Phenyl Superose fraction No. 6	0.18	41.70	7.5	10	41.7
Phenyl Superose fraction No. 7	0.21	35.70	7.5	10	35.7

Purification procedures are described in Methods. Hydrophobic interaction chromatography was performed in a Phenyl Superose HR5/5 column; the start buffer was 2 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 50 mM sodium phosphate pH 7 and the limit buffer was 20% acetonitrile, 50 mM sodium phosphate, pH 7.

<sup>a</sup>Protein content is expressed in mg.

<sup>b</sup>Specific activity is expressed in estrocolonyone-I U/mg protein.

<sup>c</sup>Total estrocolonyone-I yield is calculated as the product of the protein content by the specific activity of the estrocolonyone-I preparation.

<sup>d</sup>Percent yield is calculated as  $100 \times \text{total estrocolonyone-I yield of each purification step} / \text{estrocolonyone-I yield of the starting material (BaCl}_2\text{-plasma)}$ .

<sup>e</sup>Purification is calculated as the ratio between the specific activity achieved after a purification step and the initial specific activity of the starting material.

green chromatography; the overall purification achieved was 37- to 47-fold. A representative experiment is shown in Table 4. Phenyl Superose chromatography of the MonoQ estrocolonyone-I fraction resulted in a further 10-fold purification (Fig. 4).

#### *Estrocolonyone-I isoelectric point by chromatofocusing*

HSA-free estrocolonyone-I obtained by MonoQ chromatography was chromatofocused using a 5.5 to 4.0 pH gradient. The inhibitory activity eluted at pH 4.5 to 4.8 (Fig. 5).

#### *Estrocolonyone-I molecular weight*

Chromatography of CDHuS through a Superdex-200 column resulted in coelution of HSA and estrocolonyone-I (not shown). Estrocolonyone-I purified by anion exchange chromatography eluted from a Superose 12 column at volumes corresponding to a  $M_w$  of 70–90 kDa (Fig. 6).

Comparable molecular weight was obtained using 6 M urea in the running buffer.

## DISCUSSION

We described the inhibitory effect of serum on the proliferation of both human and rodent estrogen sensitive cells [10, 11, 14–16]. Estrogens cancel this inhibitory effect by mechanisms not fully understood [16]. Herein, we describe physicochemical properties of the molecule putatively responsible for the inhibitory effect of serum, namely, estrocolonyone-I. The proliferation-inhibiting activity remained in the macromolecular compartment after extensive dialysis against 0.5 M acetic acid through membranes of a nominal cutoff = 25 kDa and was not extracted by organic solvents (Table 1). Protease treatment of unfractionated mammalian serum was hindered by endogenous protease inhibitors; removal of protease inhibitors from human

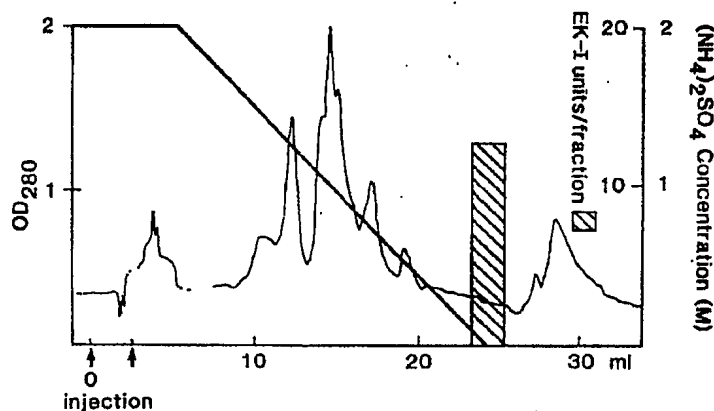


Fig. 4. 10 mg MonoQ fraction were chromatographed through a phenyl Superose-HR5/5 column. Start buffer: 2 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 50 mM sodium phosphate pH 7. Limit buffer: 20% acetonitrile, 50 mM sodium phosphate, pH 7.

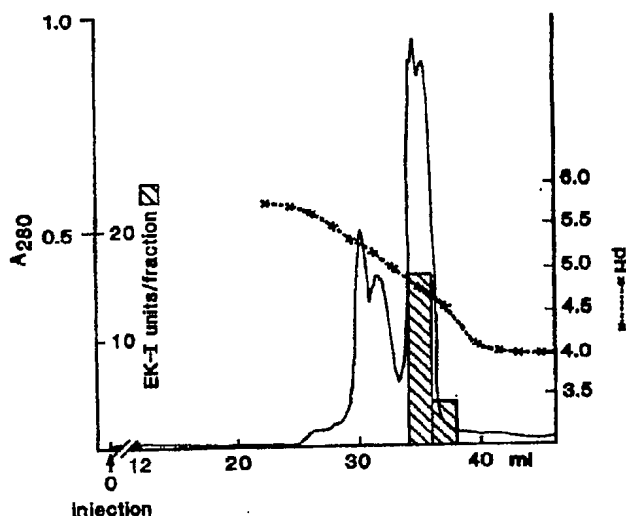


Fig. 5. Chromatofocusing profile of the MonoQ fraction. 25 mg protein were injected into a MonoP HR25/5 column equilibrated with 50 mM piperazine pH 5.5. After isocratic elution with this buffer, separation was achieved with a self generated pH 5.5-4 gradient by 10% Polybuffer pH 4.

plasma by Matrex-green chromatography followed by protease treatment completely abolished the inhibitory activity (Table 2). Trypsin treatment of chicken serum, which lacks anti-trypsin activity, significantly reduced estrocolonyone-I activity. Reduction of disulfide bonds by DTT destroyed estrocolonyone-I activity only when performed in the presence of denaturing concentrations of urea, suggesting that either critical disulfide bonds are not accessible in the native conformation or that they regenerate correctly only in this state. These results strongly suggest that estrocolonyone-I is not a TGF- $\beta$ -like molecule, since TGF- $\beta$  activity is irreversibly destroyed by 10-100 mM DTT. Moreover, TGF- $\beta$  did not significantly inhibit the proliferation of MCF<sub>7</sub> cells. This is in agreement with the results reported by Laursen

*et al.* [30]. Acid extraction was performed to determine whether or not estrocolonyone-I was a chalone-like oligopeptide; these experiments confirmed the macromolecular nature of estrocolonyone-I activity. These data suggest that estrocolonyone-I is a polypeptide; henceforth, protein purification methods were used for estrocolonyone-I characterization.

(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> precipitation is often used to reduce the contaminant burden to the following chromatographic step. Estrocolonyone-I and HSA coprecipitated with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; because HSA is the most abundant plasma protein, this fractionation procedure was ruled out. These two moieties also coeluted by Cibacron blue chromatography. To determine whether or not the inhibitory activity of serum was due to albumin [30], the inhibitory activity of HSA

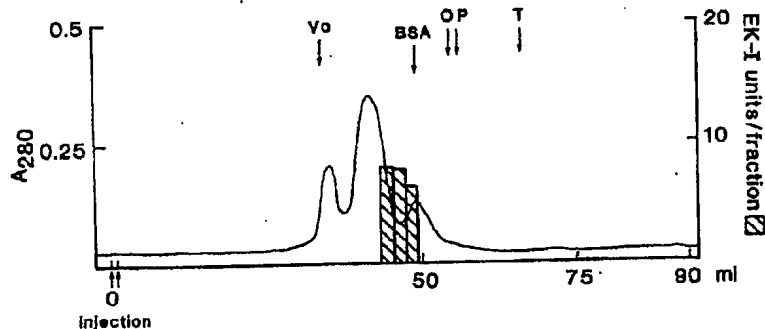


Fig. 6. Gel filtration profile of the MonoQ fraction. 30 mg protein (2 ml) were injected into a Superose 12 HR16/50 equilibrated with 100 mM NaCl, 50 mM BTP pH 7. Arrows indicate the elution of molecular weight markers. V<sub>0</sub>: void volume (blue dextran), BSA: bovine serum albumin (66 kDa), O: ovalbumin (45 kDa), P: pepsin (34.7 kDa) and T: trypsinogen (24 kDa).

(Table 1) and of serum from an analbuminemic donor were assessed (Fig. 2). These results indicated that estrocolyone-I activity was not due to HSA.

HSA was removed by adsorption to Matrex-green. Estrocolyone-I isoelectric point was 4.5-4.8; the native molecular weight was 70-80 kDa. Both results were obtained using chromatographic techniques; since estrocolyone-I detection relies on a bioassay, the use of analytical gel electrophoresis and isoelectric focusing is precluded. Partial purification was obtained by HIC and anion exchange chromatography of the Matrex-green fraction. Substantial purification was achieved by using Matrex-green to remove HSA, followed by anion exchange chromatography and HIC. These results suggest that (1) estrocolyone-I is a protein, and (2) its inhibitory effect is not due to mere estrogen trapping since SHBG and albumin fail to inhibit MCF<sub>7</sub> cell proliferation (Table 1).

Finally, these data strengthen the notion that a plasma-borne factor, estrocolyone-I, is responsible for the serum-induced inhibition of the proliferation of estrogen-sensitive MCF<sub>7</sub> cells. Further purification of estrocolyone-I is being pursued, a step necessary for a mechanistic understanding of its action and that of estrogens on the control of cell proliferation.

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